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Pseudomonas aeruginosa lasR mutants are associated with cystic fibrosis lung disease progression☆

Lucas R. Hoffman [a,*], Hemantha D. Kulasekara [b], Julia Emerson [a], Laura S. Houston [a], Jane L. Burns [a], Bonnie W. Ramsey [a], Samuel I. Miller [b,c,d]

[a] *Department of Pediatrics, University of Washington School of Medicine, Seattle, WA 98195, United States*
[b] *Department of Microbiology, University of Washington School of Medicine, Seattle, WA 98195, United States*
[c] *Department of Medicine, University of Washington School of Medicine, Seattle, WA 98195, United States*
[d] *Department of Genome Sciences, University of Washington School of Medicine, Seattle, WA 98195, United States*

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Abstract

Background: Pseudomonas aeruginosa with mutations in the transcriptional regulator LasR chronically infect the airways of people with cystic fibrosis (CF), yet the prevalence and clinical implications of *lasR* mutant infection are unknown.
Methods: In an exploratory study, we screened 166 *P. aeruginosa* isolates from 58 CF patients for LasR inactivation and mucoidy, and compared clinical characteristics among source patients.
Results: lasR mutation prevalence was comparable to that of mucoidy, the best-described CF-adapted phenotype, but affected patients were on average approximately 2 years younger. In a regression analysis, lung function decline with age was worse among patients with *lasR* mutant infection than in those without, similar to the effect of mucoidy.
Conclusions: Culture positivity for *lasR* mutant *P. aeruginosa* may serve as a marker of early CF adaptive change of prognostic significance. Furthermore, as LasR inactivation alters susceptibility to antibiotics, infection with *lasR* mutant *P. aeruginosa* may impact response to therapy.

Keywords: Cystic fibrosis; *Pseudomonas aeruginosa*; *lasR*; Mucoidy; Lung function

1. Introduction

Microbes that cause chronic infections undergo genetic change as they adapt to selective pressures encountered in host tissues. For example, in the chronic airway infection in people with cystic fibrosis (CF), *P. aeruginosa* undergoes multiple genetic changes [1]. The best described of these changes results in the over-production of exopolysaccharide, or mucoidy (usually due to mutations in *mucA* or related genes), a phenotype observed commonly among CF isolates [2]. Infection with mucoid *P. aeruginosa* increases in prevalence with advancing age among CF patients, and is associated with accelerated lung function decline compared with infection with non-mucoid isolates [3].

Recently, another *P. aeruginosa* adaptive mutation was found to occur during CF infections: inactivation of the transcriptional regulator LasR [1,4–8]. Null mutation of the *lasR* gene leads to several phenotypic changes of potential clinical significance, including a growth advantage in amino acids abundant in CF airway secretions [8,9]. *lasR* mutation leads to a distinctive colony morphology that includes surface iridescent sheen and colony flattening, the latter due to cell autolysis (Fig. 1). These colony characteristics facilitate the identification of *lasR* mutant isolates [1,8]. *lasR* mutants also exhibit increased β-lactamase activity [8]. Furthermore, quorum sensing blockade by chemical inhibitors (while not specific for the *las* system) has been shown to alter

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* Corresponding author. University of Washington School of Medicine, Children's Hospital and Regional Medical Center, 4800 Sand Point Way NE, Mailstop A-5937, Seattle, WA 98195, United States. Tel. +1 206 987 2174.
E-mail address: lhoffm@u.washington.edu (L.R. Hoffman).

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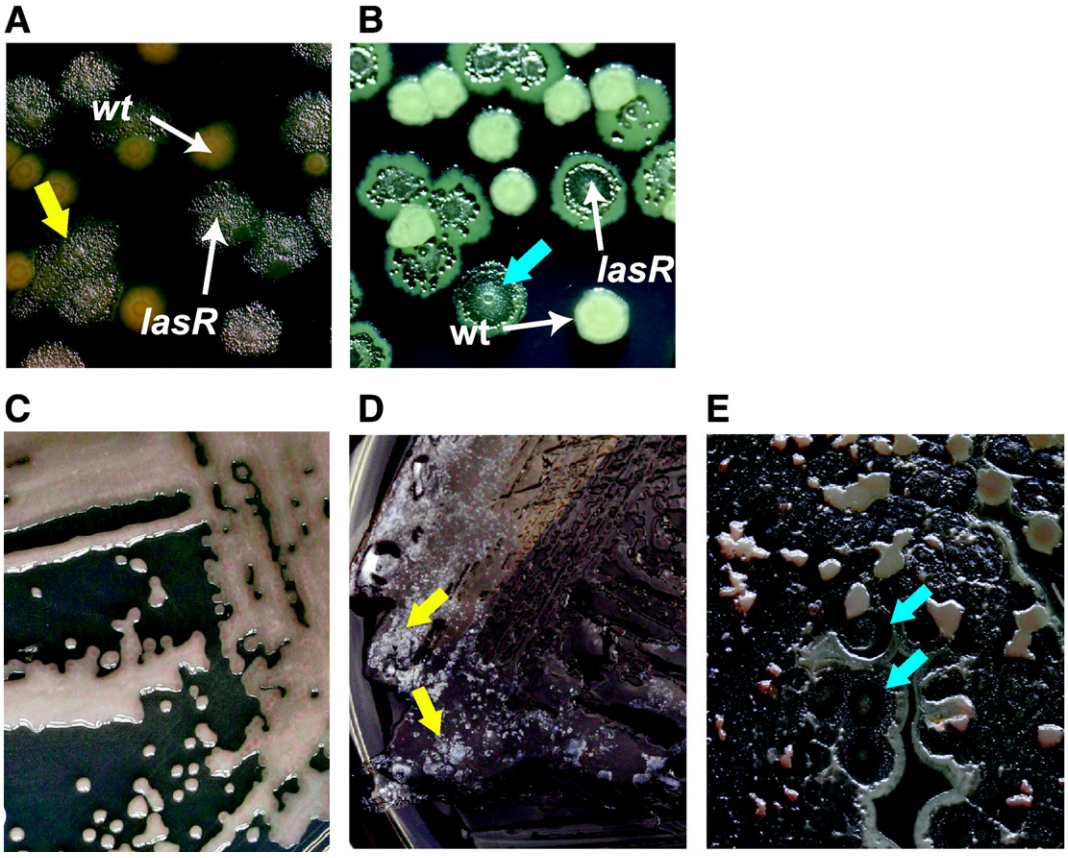

Fig. 1. Characteristic colony morphology identifies *lasR* mutant *P. aeruginosa* CF isolates. (A) LB agar-grown colonies of two clonally-related clinical isolates of *P. aeruginosa* from a single CF patient, one with an inactivating *lasR* mutation and the other with wild-type *lasR*, as indicated. The *lasR* mutant isolate displays phenotypes characteristic of *lasR* mutants, including surface iridescent, metallic sheen, which is particularly evident in this photograph (yellow arrow). (B) Another pair of LB agar-grown, clonally-related clinical *P. aeruginosa* isolates in which one isolate has an inactivating *lasR* mutation that demonstrates colony lysis and flattening (blue arrow). (C) An LB agar-grown *P. aeruginosa* isolate that is mucoid and has wild-type LasR protein function, for comparison with (D–E). (D) A mucoid *P. aeruginosa* isolate with an inactivating *lasR* mutation, exhibiting floating, iridescent material (yellow arrow). (E) Another mucoid isolate with inactivating *lasR* mutation in which colony lysis is particularly evident (blue arrow).

susceptibility to other biocides and antibiotics [11]. Thus, *lasR* mutation could impact both the effectiveness of antibiotic treatments for CF lung infection and the course of CF lung disease by resulting in an increased growth rate within the airway, where amino acids are abundant [9].

Therefore, we hypothesized that infection with *lasR* mutant *P. aeruginosa* is common and resulted in an association with a more severe course of CF lung disease, similar to the association with the mucoidy phenotype. To investigate this hypothesis, we performed an exploratory, cross-sectional, retrospective investigation of the prevalence of *lasR* mutant isolates among children attending the Children's Hospital and Regional Medical Center (CHRMC) CF clinic in Seattle, and compared age and lung function values among these patients.

2. Methods

2.1. Isolates and patients

P. aeruginosa isolates were selected at random from the CHRMC Antimicrobial Toolkit, a large archive of bacteria from local CF patients maintained for research. Isolates were eligible for selection if obtained from patients who met the following criteria: attendance at the CHRMC CF clinic during the years 1988–2002, age 15 years or younger during the study period, documentation of informed consent for studies involving clinical bacterial isolates and linked data, and culture positivity for archived *P. aeruginosa*. 58 patients were selected according to these criteria. Patient age and, where available, lung function parameters including forced expiratory volume in 1 s (FEV_1) were recorded from the culture dates on which cultures were performed. Data regarding initial culture positivity (required to define duration of infection) with *P. aeruginosa* were not reliably available for this patient population. The conduct of this study was approved by the CHRMC Human Subjects Institutional Review Board (IRB).

2.2. Isolate phenotypic analysis

Each isolate was assigned a study code. Colony surface iridescent sheen and autolysis were analyzed as described [8], and mucoidy was identified by standard visual inspection [2,12], in parallel by three separate microbiologists, including one clinical microbiologist, all of whom were unaware of the culture dates or source patient identities.

2.3. Isolate genotypic analysis

The DNA sequences of the *lasR* genes from a subset of isolates were obtained as described [8]. For some isolates, data from multilocus sequence typing were available from previous analyses [1].

2.4. Statistical analysis

Data were summarized using descriptive statistics. The frequencies of detection of isolates displaying mucoidy and/or the *lasR* mutant phenotype were determined for the entire isolate set, and the frequencies of cultures positive for isolates displaying each phenotype were also described. Mean age of earliest culture positivity for mucoid or *lasR* mutant isolates was calculated for the study population. Linear regression models were used to examine the cross-sectional relationship between lung function and age. These models included age-phenotype interaction terms to allow estimation of separate slopes for lung function decline with age for each phenotype. Three separate regression models were fit to the data for patients from whom concurrent culture and lung function results were available. In each model, slope and 95% confidence intervals were estimated for the decline in FEV_1 percent predicted for each year of age. Several patients had cultures available from two visits; thus, regression models accounted for repeated observations per patient. If a given culture yielded multiple *P. aeruginosa* isolates, the patient was classified as having a sheen positive (i.e., *lasR* mutant) or mucoid positive culture for that date if at least 1 isolate displayed the characteristic of interest. All analyses were performed using Stata, version 9.1.

3. Results

3.1. Study population and colony phenotypes

We examined 166 *P. aeruginosa* isolates (from 58 patients; average 2.8 isolates per patient, range 1–7) randomly selected from a large archive of isolates collected from young CF patients during routine clinical care. Characteristics of the study population are detailed in Table 1; descriptions of the isolates, their phenotypes, and genotypic information (when available) are included in Supplemental Table 1. We screened this isolate collection for colony surface iridescent, metallic sheen, a phenotype that specifically identifies *lasR* mutation, including in the presence of mucoidy [8] (Fig. 1). We confirmed this 100% predictive relationship by fully sequencing *lasR* from 17 sheen-positive and 44 sheen-negative isolates (of which 9 and 25 were mucoid, respectively — results shown in Supplemental Table 1). Therefore, isolates with sheen will hereafter be referred to as "*lasR* mutant", while those without sheen will be referred to as "*lasR* wild-type". The presence or absence of mucoidy (which is not caused by *lasR* mutation) was also described for each isolate. Colony phenotypes were described independently by three researchers; in cases of disagreement regarding phenotypes, the majority decision prevailed. All three observers agreed on the presence of sheen, indicative of LasR inactivation, or its absence for 98% of isolates, and on the presence or absence of mucoidy for 96% of isolates. In this collection, the presence of sheen was observed among 52 of 166 (31%) isolates, a prevalence comparable to that we observed for mucoidy (62 of 166 isolates, or 37%).

Table 1
Characteristics of the study population are shown

	N (%)
Males	31 (53.5%)
Females	27 (46.5%)
Genotype: ΔF508 homozygous	35 (60.3%)
Genotype: ΔF508 heterozygous [a]	20 (34.5%)
Genotype: other [b]	1 (1.7%)
Genotype: missing	2 (3.5%)
Isolates: *lasR* mutants	52/166 (31%)
Isolates: Mucoid	62/166 (37%)
Isolates: *lasR* mutant and mucoid	19/166 (11%)
Isolates: *lasR* wild-type and non-mucoid	71/166 (43%)
	Mean (SD) [min, max]
Age (years) at earliest culture date	8.5 (4.2) [0.3, 14.9]
Among patients with FEV_1 data available (N=44 patients, 52 culture dates)	
Age (years) at earliest culture date	10.6 (2.7) [5.8, 14.7]
FEV_1 % predicted at earliest culture date	79.2 (20.9) [27, 127]

The full dataset included 166 *P. aeruginosa* isolates representing 85 culture dates and 58 patients.

[a] Includes 6 patients with one ΔF508 mutation and one mutation that was not identified. ΔF508 is the CF disease-causing mutation that is most common and is associated with a relatively severe phenotype [14].

[b] Includes 1 patient with one non-F508 mutation and one mutation that was not identified.

The *lasR* sequencing data in Supplemental Table 1, coupled with multilocus sequence typing analysis of a subset of these isolates described previously [1] and the wide diversity of plate phenotypes of *lasR* mutants studied (not shown) indicated that *lasR* mutant isolates among this population did not represent a single, clonal (epidemic) strain of *P. aeruginosa*.

3.2. Age of source patients with specific isolates

Using the earliest positive culture per patient, average age at culture positivity for *lasR* mutant isolates was 9.2 years (SD 4.4), slightly younger than the 11.0 years (SD 3.5) we observed for mucoidy. These results suggest that *lasR* mutation may arise earlier than mucoidy during adaptation to CF airways.

3.3. Impact of LasR inactivation on lung function

We then performed an exploratory analysis of the relationship between lung function (indicated by forced expiratory volume in 1 s, FEV_1) and age using linked clinical data for all patients for whom such data were available (44 patients, 52 cultures, 105 isolates). We sought to determine how this relationship differed according to patients' *lasR* or mucoidy status on a given culture date. The 52 culture dates analyzed included 35 cultures with only *lasR* wild-type isolates and 17 cultures with *lasR* mutant isolates detected. As shown in Table 2, the presence of infection with *lasR* mutant *P. aeruginosa* isolates was associated with a statistically significant negative slope for FEV_1 percent predicted versus age, in contrast to the slope for presence of

Table 2
Slopes, 95% confidence intervals, and *P*-values computed from regression models for the relationship between FEV_1 percent predicted and age according to culture phenotype status as shown

Group	Slope (decline in FEV_1% predicted per year of age)	95% CI for slope	*P*-value
lasR mutant [a] *P. aeruginosa*	−4.1	(−7.0, −1.2)	0.006
lasR wild-type [a] *P. aeruginosa*	−2.3	(−4.6, −0.02)	0.05
Mucoid *P. aeruginosa*	−4.0	(−6.1, −1.9)	<0.001
Non-mucoid *P. aeruginosa*	−0.3	(−2.7, 2.1)	0.80
All subjects combined	−2.9	(−4.5, −1.3)	0.001

Results were not corrected for multiple comparisons.
[a] Colony surface sheen was used as a surrogate marker for LasR inactivation [8].

only *lasR* wild-type isolates, which was less negative and similar to the slope for the entire collection. This difference in slopes was similar to that calculated for the presence of mucoid isolates relative to the presence of only non-mucoid isolates (Table 2). One potential explanation for the difference in the slopes for cultures with *lasR* wild-type isolates, when compared with cultures with non-mucoid isolates, was a higher co-prevalence of mucoidy among *lasR* wild-type cultures (67%) compared to a lower co-prevalence of *lasR* mutants among non-mucoid cultures (33%).

4. Discussion

Our results suggest that *lasR* inactivation may be associated with progression of CF lung disease, similar to mucoidy [3], and may represent an earlier marker of poor prognosis. *lasR* mutant *P. aeruginosa* isolates have been observed from diverse clinical sources [10], including endotracheal tubes [13], and among CF patients from North America [1,8,22], Europe [21], and Australia [5,7]. In one retrospective study of isolates collected longitudinally from 30 CF patients in Seattle, *lasR* mutant isolates were found to have emerged independently among 19 (63%), in many cases without the identification of concurrent wild-type isolates. In several instances, multiple *lasR* mutations were found in the same isolates, suggesting strong selective pressure for loss of LasR function within CF airways [1,8].

Despite the considerable prevalence of *lasR* mutant isolates among CF patients, the clinical impact of these infections was not previously studied. (While our study could not establish a causal relationship between *lasR* mutant infection and clinical course, these results suggest that *lasR* mutation is at least associated with lung disease progression.) Laboratory analyses demonstrated that *lasR* mutant isolates exhibit at least two phenotypes *in vitro* that could negatively impact clinical course: they have a growth advantage using specific amino acids, such as phenylalanine, that are known to be abundant in CF secretions [8,9], and they are also relatively tolerant to β-lactam antibiotics used frequently in CF therapy, including ceftazidime [14], due to increased β-lactamase activity relative to their wild-type counterparts in all genetic backgrounds tested to date ([8] and data not shown). Unfortunately, our clinical database did not contain sufficient information regarding preceding or concurrent antibiotic treatments to examine whether patients with *lasR* mutant isolates were either more likely to have received ceftazidime or other β-lactam antibiotics prior to isolation, or whether they responded less well to such therapies, than did patients without *lasR* mutants. Regardless, these two characteristics–antibiotic resistance and high growth yield in abundant host nutrients–may help to explain the association between poor clinical course and *lasR* mutant infection among CF patients shown in Table 2, despite the fact that inactivating mutation in *lasR* would also be predicted to decrease expression of some characteristics associated with acute virulence [15]. It should be noted that some LasR-dependent products and phenotypes traditionally associated with virulence, including protease secretion and biofilm formation, were shown to be intact among many *lasR* mutant clinical isolates, suggesting that these functions may have become uncoupled from LasR regulation [5,13,22]. Another characteristic of *lasR* mutant isolates that may be of clinical significance is their relatively high susceptibility to amino acid-based metabolic inhibitors, including fluorophenylalanine [8]. This quality could be exploited to design targeted therapies when *lasR* mutant isolates are identified in clinical specimens.

The presence of *lasR* inactivating mutations was easily identifiable by screening for the unique colony sheen phenotype. We found that inter-observer reproducibility for sheen was at least as good, and likely better, than it was for mucoidy, a colony morphotype that is currently reported by clinical microbiological laboratories due to its association with poor prognosis [2,3]. Thus, the identification of *lasR* mutants is feasible for most clinical laboratories, and may be of both prognostic and therapeutic significance.

Our results suggest that *lasR* mutation may arise earlier than mucoidy, indicative of an early adaptation to the CF airway. Current CF treatment strategies focus on early eradication of *P. aeruginosa* upon initial culture positivity [14], driven in large part by the observation that adaptation of *P. aeruginosa* to the CF airway, including the development of mucoidy, is associated with a worse prognosis and recalcitrance to eradication [2,3]. Given the relative resistance of *lasR* mutants to β-lactam antibiotics [8], the current results further support this early treatment strategy. Furthermore, these observations suggest that regimens that include a β-lactamase inhibitor may be particularly beneficial upon culture positivity for *lasR* mutants. Therefore, the identification of bacterial characteristics (such as colony sheen) may prove useful in directing therapy.

Our study is limited by its retrospective and cross-sectional nature, as well as the availability of lung function data from a limited subset of study patients. Furthermore, while duration of infection (as opposed to patient age) has been associated with the emergence of mucoidy and CF lung function change [16], we have used patient age at isolation instead because data regarding the dates of initial culture positivity for *P. aeruginosa* (and thus duration of infection for the currently studied cultures) were not reliably available for this study population. In support of this approach, recent work demonstrated that culture may be

an unreliable method for defining timing of initial infection [17–19]. Regardless, the utility of *lasR* mutation, indicated by the easily identifiable sheen phenotype, as a predictor of lung disease progression should be validated in a well-designed, prospective study, preferably at multiple sites. The optimal study would also be longitudinal, in order to investigate the evolution of lung disease severity before and after isolation of *lasR* mutants. Such a study would also be useful in identifying clinical factors, such as antecedent antibiotic treatments, that are associated with the emergence of *lasR* mutant isolates. If the current results are validated in such a prospective study, the clinical identification of *lasR* mutant *P. aeruginosa* isolates from CF patients could represent a significant advance in CF clinical microbiology, perhaps with utility higher than that for either antibiotic susceptibility testing [20] or the identification of mucoidy (3).

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.jcf.2008.09.006.

References

[1] Smith EE, Buckley DG, Wu Z, Saenphimmachak C, Hoffman LR, D'Argenio DA, et al. Genetic adaptation by *Pseudomonas aeruginosa* to the airways of cystic fibrosis patients. Proc Natl Acad Sci U S A May 30 2006;103(22):8487–92.

[2] Govan JR, Deretic V. Microbial pathogenesis in cystic fibrosis: mucoid *Pseudomonas aeruginosa* and *Burkholderia cepacia*. Microbiol Rev 1996;60(3):539–74.

[3] Li Z, Kosorok MR, Farrell PM, Laxova A, West SE, Green CG, et al. Longitudinal development of mucoid *Pseudomonas aeruginosa* infection and lung disease progression in children with cystic fibrosis. Jama Feb 2 2005;293(5):581–8.

[4] Fothergill JL, Panagea S, Hart CA, Walshaw MJ, Pitt TL, Winstanley C. Widespread pyocyanin over-production among isolates of a cystic fibrosis epidemic strain. BMC Microbiol 2007;7:45.

[5] Tingpej P, Smith L, Rose B, Zhu H, Conibear T, Al Nassafi K, et al. Phenotypic characterization of clonal and nonclonal *Pseudomonas aeruginosa* strains isolated from lungs of adults with cystic fibrosis. J Clin Microbiol Jun 2007;45(6):1697–704.

[6] Heurlier K, Denervaud V, Haenni M, Guy L, Krishnapillai V, Haas D. Quorum-sensing-negative (lasR) mutants of *Pseudomonas aeruginosa* avoid cell lysis and death. J Bacteriol Jul 2005;187(14):4875–83.

[7] Schaber JA, Carty NL, McDonald NA, Graham ED, Cheluvappa R, Griswold JA, et al. Analysis of quorum sensing-deficient clinical isolates of *Pseudomonas aeruginosa*. J Med Microbiol Sep 2004;53(Pt 9):841–53.

[8] D'Argenio DA, Wu M, Hoffman LR, Kulasekara HD, Deziel E, Smith EE, et al. Growth phenotypes of *Pseudomonas aeruginosa* lasR mutants adapted to the airways of cystic fibrosis patients. Mol Microbiol Apr 2007;64(2):512–33.

[9] Barth AL, Pitt TL. The high amino-acid content of sputum from cystic fibrosis patients promotes growth of auxotrophic *Pseudomonas aeruginosa*. J Med Microbiol Aug 1996;45(2):110–9.

[10] Cabrol S, Olliver A, Pier GB, Andremont A, Ruimy R. Transcription of quorum-sensing system genes in clinical and environmental isolates of *Pseudomonas aeruginosa*. J Bacteriol Dec 2003;185(24):7222–30.

[11] Bjarnsholt T, Jensen PO, Burmolle M, Hentzer M, Haagensen JA, Hougen HP, et al. *Pseudomonas aeruginosa* tolerance to tobramycin, hydrogen peroxide and polymorphonuclear leukocytes is quorum-sensing dependent. Microbiology Feb 2005;151(Pt 2):373–83.

[12] Burns JL, Van Dalfsen JM, Shawar RM, Otto KL, Garber RL, Quan JM, et al. Effect of chronic intermittent administration of inhaled tobramycin on respiratory microbial flora in patients with cystic fibrosis. J Infect Dis 1999;179(5):1190–6.

[13] Denervaud V, TuQuoc P, Blanc D, Favre-Bonte S, Krishnapillai V, Reimmann C, et al. Characterization of cell-to-cell signaling-deficient *Pseudomonas aeruginosa* strains colonizing intubated patients. J Clin Microbiol Feb 2004;42(2):554–62.

[14] Gibson RL, Burns JL, Ramsey BW. Pathophysiology and management of pulmonary infections in cystic fibrosis. Am J Respir Crit Care Med Oct 15 2003;168(8):918–51.

[15] Hentzer M, Wu H, Andersen JB, Riedel K, Rasmussen TB, Bagge N, et al. Attenuation of *Pseudomonas aeruginosa* virulence by quorum sensing inhibitors. Embo J Aug 1 2003;22(15):3803–15.

[16] Pedersen SS, Hoiby N, Espersen F, Koch C. Role of alginate in infection with mucoid *Pseudomonas aeruginosa* in cystic fibrosis. Thorax Jan 1992;47(1):6–13.

[17] Burns JL, Gibson RL, McNamara S, Yim D, Emerson J, Rosenfeld M, et al. Longitudinal assessment of *Pseudomonas aeruginosa* in young children with cystic fibrosis. J Infect Dis Feb 1 2001;183(3):444–52.

[18] West SE, Zeng L, Lee BL, Kosorok MR, Laxova A, Rock MJ, et al. Respiratory infections with *Pseudomonas aeruginosa* in children with cystic fibrosis: early detection by serology and assessment of risk factors. Jama 2002;287(22):2958–67.

[19] da Silva Filho LV, Tateno AF, Martins KM, Azzuz Chernishev AC, Garcia Dde O, Haug M, et al. The combination of PCR and serology increases the diagnosis of *Pseudomonas aeruginosa* colonization/infection in cystic fibrosis. Pediatr Pulmonol Oct 2007;42(10):938–44.

[20] Smith AL, Fiel SB, Mayer-Hamblett N, Ramsey B, Burns JL. Susceptibility testing of *Pseudomonas aeruginosa* isolates and clinical response to parenteral antibiotic administration: lack of association in cystic fibrosis. Chest May 2003;123(5):1495–502.

[21] Salunkhe P, Smart CH, Morgan JA, Panagea S, Walshaw MJ, Hart CA, et al. A cystic fibrosis epidemic strain of *Pseudomonas aeruginosa* displays enhanced virulence and antimicrobial resistance. J Bacteriol Jul 2005;187(14):4908–20.

[22] Schaber JA, Carty NL, McDonald NA, Graham ED, Cheluvappa R, Griswold JA, et al. Analysis of quorum sensing-deficient clinical isolates of *Pseudomonas aeruginosa*. J Med Microbiol Sep 2004;53(Pt 9):841–53.